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Isolation, Characterization, and Identification of Gut Bacteria from Piscine Ectoparasite, Argulus Bengalensis (Ramakrishna 1951) and their Relationship with Haematophagy

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Abstract

Gut microbiome plays a pivotal role in health and nutrition of most organisms. Argulus bengalensis is a haematophagus ectoparasite of fresh water carp and its infection causes extensive damage to fish farms. The present study aims at isolation, characterization and identification of the gut bacteria from Argulus bengalensis and to decipher its potential contribution in haematophagy of the parasite. The gut bacteria were isolated, cultured and identified based on analysis of its morphological, physiological and biochemical features as well as 16s rDNA analyses against the NCBI genetic database. Antibiotic sensitivity was tested. Haemolytic activity and ability of the isolates to produce anticoagulant substance were also tested to justify the hypothesis. Two isolates were identified to be similar type strains of Acenatobacter baumanii and Aeromonas hydrophila respectively which have accession number MW811800 and MW806655 respectively. Both bacteria were found sensitive to most antibiotics. Both Acenatobacter baumanii and Aeromonas hydrophila showed their haemolytic activities and able to secrete anticoagulant substances establishing their mutualistic association which increase the fitness of the parasitic host facilitating haematophagy. The outcome of the study thus may provide a scientific direction to develop novel methods to control of Argulosis.

Keywords: *Argulus bengalensis; Haematophagy; Acenatobacter baumanii; Aeromonas hydrophila Haemolysis; Anticoagulant*

1. Introduction

Symbiosis, sometimes known as "living together," primarily refers to a tight relationship between several species. The "endosymbiosis" is one of the many symbiotic connections, and it exhibits the most coherent forms. In this connection, the symbiont inhabits the host, the partner it partners with. Symbiotic bacteria are typically found in species that have restricted diets, such as those that eat woody debris, vertebrate blood, or plant sap. The gut lumen, the hemocoel, or specialized cells called mycetocytes or bacteriocytes are frequently the locations of certain bacterial species in these circumstances (Buchner, 1965). Such bacteria are generally found in the host insects, are passed down vertically from generation to generation (Pais et al., 2008; Hosokawa et al., 2010), and frequently cannot be grown in vitro. Many different insect species depend on microorganisms for their growth and development. These contributions in insects include the synthesis of necessary nutrients, the generation of vitamins and sterols, the digestion of meals like cellulose, the determination of food consumption, and the detoxification of foods (Douglas, 2009). There aren't many studies that describe

the elements and roles that these interactions play, despite the importance of these microbial linkages with insects. Insects that consume diets deficient in nutrients frequently have bacterial endosymbionts. When their symbionts are experimentally removed, the host insects frequently experience stunted development, infertility, and/or death (Buchner, 1965; Douglas, 1989; Baumann and Moran, 1997).

All of the genera in the Branchiura order are parasitic, mostly on fish. According to Poly (2008), there are about 129 species of Argulu (MÜller, 1785) fish that are known to be haematophagous (eating blood) eaters (Swanepoel and Avenant-Oldewage, 1992; Gresty et al., 1993; Saha et al., 2011). The endosymbionts replenish vital vitamins for the survival of their hosts in the nutrient-limited diets of blood-feeding insects (Pais et al., 2008; Hosokawa et al., 2010). Haematophagy has developed as a favored method of eating in many tiny creatures, such as worms and arthropods, since blood is a fluid tissue rich in nourishing proteins and fats that can be ingested without requiring a great deal of effort. Argulus feeds by penetrating the skin with a lengthy stylet structure that resembles a spine. The parasite uses two chemicals that are released from glands associated with the feeding apparatus as anti-haemostatic agents: a vasodialator (Marshall et al., 2008) and an anticoagulant (Saha et al., 2011). Despite their diversity, some other animals that only consume blood, such as leeches, hookworms, and various species of vampire bats, all secrete anti-haemostatic and fibrinolytic substances from their salivary glands to stop blood clotting while they are feeding and blood storage in the gut while digesting (Ribeiro, 1987; Fontaine, et al., 2011; de Araújo, et al., 2012; Kotál, et al., 2015; Chmelař, et al., 2016). There are several anti-coagulant and anti-thrombotic compounds found in blood-sucking insects. Blood-feeding creatures form symbiotic relationships with the bacteria in their stomach, which is consistent with haematophagy. By providing extracellular enzymes, the symbionts are hypothesized to either assist the parasite's anti-haemostatic mechanism in preventing blood from coagulating in their intestines or facilitate blood digestion. The symbionts occasionally provide dietary supplements by manufacturing vitamins. In their gastrointestinal systems, all vertebrates and many invertebrates have microbial communities (Trust and Sparrow, 1974; Lindsay and Harris, 1980; Lesel et al., 1986). As a result of the host animal's digestion secretions and meal absorption, these populations increase. The bacterial flora of endogenous sources often possesses a considerable and diverse range of enzymatic abilities. It is reasonable to expect that a considerable amount of the partner's metabolism may be considerably disrupted by the enzyme mass stuck in the digestive system.

The symbionts undoubtedly have a part in certain elements of nutrition in the broadest sense, but their effects on morphology, reproduction, and digestion further complicate matters. The connection is so strong and intimate that frequently the processes involved resemble cell organelles more than they resemble those of independent bacteria. As we proposed in Ghoshal (2018), the objective of the current work is to identify and characterize the endosymbiotic bacteria and to ascertain their role in the survival of the parasite Argulus. Due to its widespread damage, argulosis is a big worry for fish growers in India. Therefore, studying the physiological importance of the symbionts is important to understand the link that may offer a clear hint to an alternate parasite management strategy.

The remainder of the essay is structured as follows. The results are presented structurally following an explanation of the study's materials and procedures. The next part covers the conclusions and wraps up the study.

2. Material and Methods

Sample Collection

Carp remains from the "Barasagar Dighi" fish farm in Malda, India (24o58'08.86" N, 88o06'09.70" E), were examined for parasitic variants of the *Argulus bengalensis* species. Cirrhinus mrigala, one of the parasite's favoured hosts, was used to grow a breeding colony of the parasite in lab settings (Hamilton, 1822). Ramakrishna (1951)'s morphometric features were used to identify the parasite.

Isolation of gut microbes from Argulus bengalensis

Ten numbers of adult *Argulus bengalensis* specimens were kept on starvation for 4 hours to clear their alimentary tract and allochthonous gut flora. Inside a laminar flow hood, the starved specimens were subjected to surface sterilization with 0.01% mercuric chloride. Thereafter the specimens were

autopsied under an inverted microscope inside the laminar hood to obtain their gut. Finally, the tissue is homogenized in a sterile condition. After five sequential 1:10 dilutions, the homogenate was employed as inoculums (Beveridge et al., 1991). In triplicate, 0.1 ml of the inoculum from each dilution was placed onto Tryptone Soya Agar (TSA) plates that had been sterilized. For the purpose of creating facultatively aerobic or anaerobic bacterial colonies, the culture plates were incubated at 30°C for 24 hours. To create pure cultures, the well-separated colonies were streaked individually on TSA plates.

Microscopic, biochemical, and molecular characterization of the microbes

Microscopic characterizations of the selected microbial strains were done after Gram staining. According to Bergey's Manual of Determinative Biology (Holt et al., 1994), biochemical characterizations were carried out. The strains' capacity to flourish at pH levels ranging from 4 to 9 was also examined, NaCl concentrations of 2% to 9%, and temperature conditions of 10ºC to 55ºC. The fermentative behavior was determined for various carbohydrates (Glucose, Mannitol, Sucrose Rhamnose, and Xylose).

Before the bacterial culture was transferred to a 2 ml microfugetube for DNA separation of the microbial isolates, it was homogenized with 1 ml of extraction buffer. After being gently blended with an equal volume of phenol, chloroform, and isoamyl alcohol (25:24:1), the homogenate was centrifuged for 15 minutes at 14000 rpm. The supernatant was collected after a second centrifugation using isoamyl alcohol (24:1) and mixed with an equal volume of chloroform. The DNA was centrifuged under the same conditions after being precipitated for 15 minutes at room temperature with the addition of 0.1 volume of 3M Sodium acetate pH 7.0 and 0.7 volume of isopropanol. The DNA pellet was washed twice with 70% ethanol and then briefly with 100% ethanol, and then dissolved in TE (Tris-Cl 10 mM pH 8.0, EDTA 1 mM). After that, the DNA pellet was air dried. to remove RNA The DNA received 5 l of free RNAse A at a concentration of 10 mg/ml. For 16S rRNA gene sequencing, two colonies (CM1MG1 and CM1MG2) were selected because they stood out from other colonies. Using the primers 518F (5' CCAGCAGCCGGTAATACG 3') and 800R (5' TACCAGGGTATCTAATCC 3'), the 16S rRNA gene was amplified using PCR (Senthilraj et al., 2016). The approximately 1,400 bp purified PCR result underwent sequencing.

The two sequences were obtained and put through BLAST analysis; phylogenetically related sequences were chosen from the NCBI Gene Bank and put through multiple sequence alignment; the aligned sequences were then cut to similar nucleotide lengths and put through phylogenetic tree construction (neighbor-joining) using MEGA 6. To get accession numbers, two isolates' partial 16S rRNA sequences were deposited in the NCBI GenBank database.

Antibiotic susceptibility test

The disc diffusion technique and susceptibility test discs (HiMedia, India) were used to assess antibiotic susceptibility in accordance with the National Committee for Clinical Laboratory Standards's (2012) standards. The strains were classified as sensitive (zone diameter 20 mm), moderately sensitive (zone diameter 15–19 mm), or resistant (zone diameter 14 mm). The susceptibility of the isolates was evaluated using the antibiotics Chloramphenicol (30 g), Streptomycin (10 g), Tetracycline (30 g), Netilmicin (30 g), Ciprofloxacin (5 g), Doxycycline (330 g), Co-trimoxazole (25 g), and Nalidixic Acid (30 g).

Detection of anticoagulant property

The capillary technique was used to determine the anticoagulant property. Non-heparinized capillary tubes in standard diameters were used for this. Triclone methane sulfonate (MS-222; 50 mg/l) was used as an anesthetic to sever the caudal peduncle of Cirrhinus mrigala in order to collect fish blood samples (Stetter, 2001; Neiffer and Stamper, 2009). A time count was then started to count the clotting time (Ct). Bacteria were cultured in nutrient broth media (30ºC, pH 7, 48 hours) centrifuged (4ºC, 4000 rpm, 10 minutes) and mixed thoroughly with the blood sample by gentle shakingin a ratio of 1:1 within two 1.5 ml micro-centrifuge tubes. Un-inoculated nutrient broth kept under the same condition and mixed with blood in the same ratio was used as control. Each of the blood mixes was immediately drawn in three capillary tubes, thereafter one end was sealed with plasticine. After every

5-second interval, a small part of the filled capillary tubes was broken and two fragments were separated gently. The end time point was noted as clotting time (Ct) as soon as the blood mix forms a continuous thread of clot between the broken ends of the tube (Hoffbrand and Steensma, 2019). The procedure was replicated five times to calculate the mean timing.

For further confirmation of Ct, an APTT test was carried out. A fish blood sample was prepared as per NCCLS Document H21-A3 and mixed with culture supernatant in a ratio of 1:1. The control was the same as mentioned previously. The mixture was then discharged into an Automated Coagulometer (Synthasil APTT) by Instrumentation Laboratory, Italy, 2020; and the test was carried out as per operator manual provided by the company, and the clotting time was recorded.

Extracellular enzyme assay

The synthesis of the extracellular enzymes amylase, lipase, protease, and cellulase was assessed in the bacterial isolates. For the qualitative assessment of enzymes, selective agar media such as peptone-gelatin-agar media, starch-agar, carboxymethylcellulose (CMC)-agar, and tributyrin-agar-containing plates were utilized. Amylase (Bernfeld, 1955) and protease (Walter, 1984) activities were quantified based on the findings of a qualitative experiment and represented as units (U). It is possible to get a more thorough explanation of the quantitative measurement of these extracellular enzymes elsewhere (Bairagi et al., 2002).

Haemolysis assay

Minor adjustments were made to the method published by Vaughan et al. (2005) for the haemolytic test of bacterial isolates. The bacteria were isolated and cultured for 48 hours at 37°C on sheep blood agar base plates (HiMedia) containing 5% sheep blood. Bacterial colonies were classed as either haemolytic (having a greenish halo around the colony), non-haemolytic (having no halo around the colony), or hemolytic (having a clear zone surrounding the colony) based on whether or not they had a clear zone.

3. Results and Discussion

Isolation

On the plate, several autochthonous bacterial colonies were discovered. These colonies were distinguished by their colony morphology, such as form, size, color, margin opacity, elevation, etc. To produce pure culture for additional research, morphologically different and dominant colonies were chosen for repeated subculture by streaking on nutrient Agar plate.

Microscopic, biochemical, and molecular characterization of the microbes

The outcomes of morphological, biochemical, and physiological characterization of the bacterial strains are depicted in Table 1. The two isolates viz CM1MG1 and CM1MG2 were Gram-negative, rod-shaped bacteria, and showed positive growth at ≤15ºC, 2–9% NaCl concentration (w/v), and between pH range of 5-9. The isolates were non-motile. Starch and gelatin were hydrolyzed by CM1MG2 only. Further CM1MG2 showed positive results for, the Voges-Proskauer, Indole, and Methyl red tests but CM1MG1 had inverse results for these. CM1MG1 showed positive results for glucose, raffinose, galactose, and xylose but negative for rest whereas CM1MG1 showed acid production from, glucose, sucrose, lactose, and fructose, but negative for rhamnose, xylose.

Table 1: Morphological and Biochemical Characteristics of both the Isolates

Characteristic	CM1MG1	CM1MG2
	Morphological	
Configuration	round	round
Margin	entire	entire
Elevation	raised	raised
Pigment	white	shiny
Density	opaque	Transparent and glossy
Gram reaction	-ve	-ve rods

	rods	
Spore	-ve	-ve
Motility	-ve	-ve
	Growth at different temperature	
10°C	-	-
15°C	-	-
25°C	+	+
30°C	+	+
37°C	+	+
42°C	+	+
50°C	-	+
55°C	-	+
	Growth at different pH	
pH4.0	-	+
pH5.0	+	+
pH6.0	+	+
pH7.0	+	+
pH8.0	+	+
pH9.0	+	+
	Growth at different concentration of NaCl %	
2.0%	+	+
3.0%	+	+
5.0%	+	+
7.0%	+	+
8.0%	+	+
9.0%	+	+
	Biochemical tests	
GrowthonMacConkey agarmedium	+	-
Indoletest	-	+
Methylred test	-	+
Voges Proskauer test	-	+
Citrate test	-	+
Casein test	-	-
Starch hydrolysis	-	+
Gelatin hydrolysis	-	+
Nitrate reduction	-	+
Catalase	+	+
Oxidase	-	+
Esculine Hydrolysis	-	+
H_2Sgasproduction	-	+
Urease test	-	-
	Acid production from carbohydrates	
Glucose	+	+
Fructose	-	+
Salicin	-	-
Mannitol	-	+

Raffinose	+	+
Sucrose	-	+
Rhamnose	-	-
Galactose	+	-
Innositol	-	-
Xylose	+	-
Maltose	-	+

Signs + and – indicate positive and negative respectively

Molecular characterization and phylogenetic tree construction

Based on the evolutionary study of the 16S rRNA gene sequence and the homology of the nucleotide sequence, it reveals that the strains CM1MG1 and CM1MG2 were 99.81% and 99.71% similar to the type strains *Acenetobacter baumanii* (LN611355.1.) and *Aeromonas hydrophila* (NR_074841.1) respectively. Type strains had been retrieved from NCBI GenBank. The dendrograms show the evolutionary relationship between two chosen strains and their closely related type strains that were obtained from NCBI GenBank (Figure 1). Submission of the sequences of the isolates to NCBI GenBank provided the accession numbers MW811800 and MW806655 respectively.

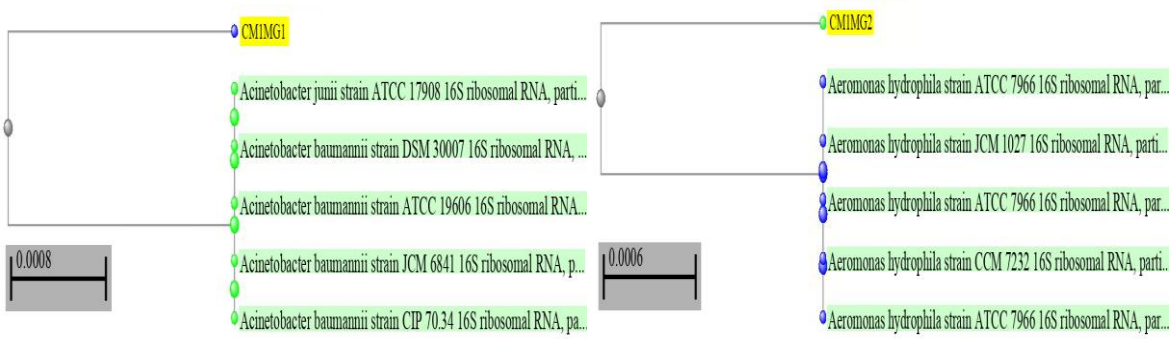

Figure 1: Dendogram of the two isolated strains, CM1MG1 and CM1MG2, displaying their phylogenetic relationships to other closely related type strains received from NCBI GenBank.

Antibiotic sensitivity test

The results of the antibiotic tests are mentioned in Table 2. The CM1MG1 was found to be sensitive to antibiotics like tetracycline, ciprofloxacin, and doxycycline, whereas moderately sensitive to co-trimoxazole and streptomycin. But CM1MG2 was found sensitive to all the discs.

Table 2: Sensitivity of different drugs on both isolates

Chemical	Disc Content (µg)	CM1MG1	CM1MG2
Chloramphenicol	30	R	S
Streptomycin	10	R	M
Tetracyclin	30	M	S
Netilimycin	30	M	M
Ciprofloxacin	5	S	S
Doxycycline	30	S	M
Cotrimoxazol e	25	M	S
Nalidixic acid	30	R	M

S = succeptible, M = moderately succeptible, R = Resistant

Anticoagulant assay

It was discovered that the mean clotting time of the blood sample combined with culture supernatant was longer than that of the control blood sample. The blood combined with CM1MG1 and CM1MG2 had mean clotting times of 15.8 and 21.6 seconds, respectively, compared to the control blood's mean clotting time of 11.8 seconds. The APTT test also revealed that the clotting time was delayed by

around 2 seconds and 30 seconds in the presence of CM1MG1 and CM1MG2 respectively, concerning the control blood sample. The results are shown in Figure 2.

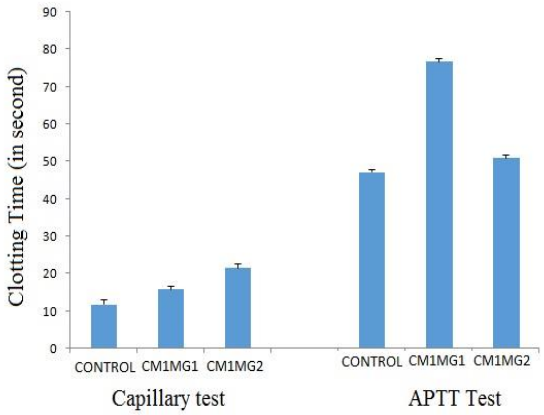

Figure 2: Effect on the clotting time of blood by two isolates CM1MG1 and CM1MG2

Extracellular enzyme assay

The lipase and cellulase activities were beyond the level of detection in both isolates. CM1MG1 exhibited amylase activity and it was 3.06±0.14 mg/ml/hr, whereas CM1MG2 exhibited protease activity and it was 0.87±0.02 µg/ml/min. But the value of protease activity in CM1MG1 and amylase activity in CM1MG2 were not of much significance.

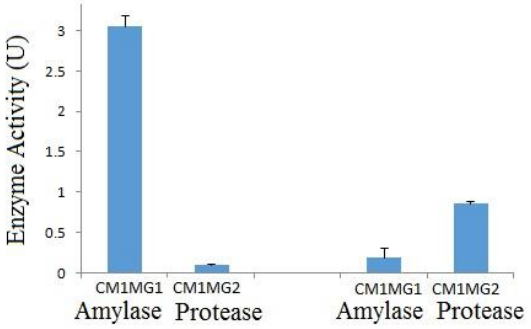

Figure 3: Results of the enzymatic activities of both the isolates

Haemolysis assay

Because CM1MG1 and CM1MG2 both had a distinct halo zone surrounding the colonies they had grown on sheep blood agar medium plates, they were both hemolytic in nature.

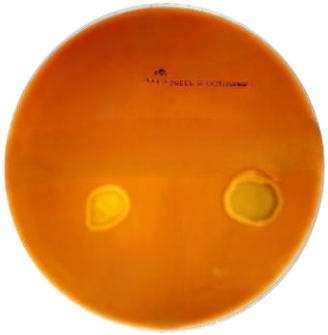

Figure 4a: *Haemolysis in CM1MG1*

Figure 4a: *Haemolysis in CM1MG2*

Figure 4: Haemolysis on blood agar plate by CM1MG1 and CM1MG2 respectively

Symbiotic associations have been overwhelmingly emphasized in the role of endosymbionts and more specifically of gut microbiota in the host nutrition in the existing literature (Clark et al., 1992; Douglas and Prosser, 1992; Kaya and Gaugler, 1993). By secreting digesting enzymes and adding vitamins, the nutritional contributions of the symbiont frequently enable the affected host to thrive on less-than-ideal diets. For endosymbionts like Buchneraspp, these nutritional contributions are well documented (Douglas, 1998). But in many instances, the bacterial population in the gut could offer comparable advantages.

The *Argulus sp* used in this investigation were fasted for 4 hours before having their gut microflora isolated. As a result, it may be assumed that these isolates are part of the autochthonous adherent microflora, as claimed by Ghosh et al. (2010). In addition, culture-based investigation of the leech gut microbiome revealed that Aeromonas sp, a haematophagous symbiont, is an exclusive and persistent symbiont (Nelson and Graf, 2012). The study also shows that two bacterial isolates may survive in a wide pH range, supporting the use of the gut as an appropriate biotope. Almost exclusively free-living, acinetobacter is frequently seen in the water or on the skin of animals (Doughari et al., 2011). *Argulus* sp frequently uses these habitats for egg-laying, larval hatching, and resting. Therefore, environmental sharing may facilitate the bacteria's connection with *Argulus*. Kar et al. (2008) claim that mannose, xylose, raffinose, and cellulose may be used by gut bacteria that produce enzymes. Therefore, the results of both isolates' carbohydrate consumption indicate that both isolates are enzyme-producing gut bacteria that can function on a variety of substrates.

Besides the results of the antibiotic susceptibility test may also provide a reference for potential treatment for *Argulus* infection for fish. The inhibition of the clotting of blood is an important adaptation associated with haematophagy (Schofield et al., 1993; Gomes et al., 2005). Anticoagulant assay of the bacteria shows anticoagulant properties as the clotting time gets delayed in the presence of culture supernatant. Similar instances were reported by Bykowska et al. (1985) and El Akoum et al. (1987) about the production of substances possessing anticoagulant properties by various bacteria like *Staphylococcus epidermis* and *Myxococcus xanthus* respectively. As *Argulus bengalensis*

predominantly feeds on tissue fluid and blood of the fish host (Saha et al., 2011) it may be apprehended that these bacteria facilitate in blood feeding and digestion. A positive result of protease and amylase activity of both the isolates respectively shows contribution in the digestion of blood.

As mentioned earlier *Argulus* primarily feeds on blood and tissue fluid which is a protein-rich diet. Minard et al. (2013) revealed the contribution of *Acinetobacter baumanii* in digesting various components specific to blood in the mosquito gut. Whereas *Aeromonas sp* is found as digestive tract symbionts of varied blood-feeding organisms including mosquitoes, medicinal leech, and the vampire bat (Pinus and Müller, 1980; Graf, 1999, 2000; Pidiyar *et al*., 2002). Taking into consideration, that the important step of blood digestion is the release of hemoglobin, the major blood protein, it was hypothesized that the gut bacteria may have a putative functional role in lysis of RBC. The hemolytic ability of both isolates marks their potential contribution to blood digestion. A mutualistic relationship of these strains with their partner thus can be validated in future studies.

4. Conclusion

So, based on the study objectives which include identifying and characterizing the endosymbiotic bacteria and determining how they contribute to the parasite Argulus' survival, it can be concluded that two isolates were found to be comparable type strains of Acenatobacter baumanii and Aeromonas hydrophila, and their mutual connection increases the fitness of the parasitic host and facilitates haematophagy, was demonstrated by both organisms' hemolytic activity and ability to secrete anticoagulant compounds. So, the study findings might offer a scientific direction for developing cutting-edge techniques to reduce argulosis.

References

Bairagi, A., Ghosh, K. S., Sen, S. K., & Ray, A. K. (2002). Enzyme producing bacterial flora isolated from fish digestive tracts. *Aquaculture international*, *10*, 109-121.

Baumann, P., & Moran, N. A. (1997). Non-cultivable microorganisms from symbiotic associations of insects and other hosts. *Antonie van Leeuwenhoek*, *72*, 39-48.

Bernfeld, B. (1955). Amylases α and α. *Methods Enzymol.*, *1*, 149-158.

Beveridge, M. C. M., Sikdar, P. K., Frerichs, G. N., & Millar, S. (1991). The ingestion of bacteria in suspension by the common carp Cyprinus carpio L. *Journal of Fish Biology*, *39*(6), 825-831.

Buchner, P. (1965). Endosymbiosis of animals with plant microorganisms. New York, N.Y.

Bykowska, K., Ludwicka, A., Lopaciuk, S., & Kopeć, M. (1985). Anticoagulant properties of extracellular slime substance produced by Staphylococcus epidermidis. *Thrombosis and haemostasis*, *54*(08), 853-856.

Chmelař, J., Kotál, J., Karim, S., Kopacek, P., Francischetti, I. M., Pedra, J. H., & Kotsyfakis, M. (2016). Sialomes and mialomes: a systems-biology view of tick tissues and tick–host interactions. *Trends in parasitology*, *32*(3), 242-254.

Clark, M. A., Baumann, L., Munson, M. A., Baumann, P., Campbell, B. C., Duffus, J. E., ... & Moran, N. A. (1992). The eubacterial endosymbionts of whiteflies (Homoptera: Aleyrodoidea) constitute a lineage distinct from the endosymbionts of aphids and mealybugs. *Current Microbiology*, *25*, 119-123.

de Araújo, C. N., Bussacos, A. C., Sousa, A. O., Hecht, M. M., & Teixeira, A. R. (2012). Interactome: smart hematophagous triatomine salivary gland molecules counteract human hemostasis during meal acquisition. *Journal of proteomics*, *75*(13), 3829-3841.

Doughari, H. J., Ndakidemi, P. A., Human, I. S., & Benade, S. (2011). The ecology, biology and pathogenesis of Acinetobacter spp.: an overview. *Microbes and environments*, *26*(2), 101-112.

Douglas, A. E. (1998). Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria Buchnera. *Annual review of entomology*, *43*(1), 17-37.

Douglas, A. E. (2009). The microbial dimension in insect nutritional ecology. *Functional Ecology*, *23*(1), 38-47.

Douglas, A. E., & Prosser, W. A. (1992). Synthesis of the essential amino acid tryptophan in the pea aphid (Acyrthosiphon pisum) symbiosis. *Journal of insect physiology*, *38*(8), 565-568.

Douglas, A.E. (1989). Mycetocyte symbiosis in insects. *Biological Reviews*, 69, 409–434

El Akoum, A., Vijayalakshmi, M., Cardon, P., Fournet, B., Sigot, M., & Guespin-Michel, J. F. (1987). Myxococcus xanthus produces an extracellular glycopeptide that displays blood anticoagulant properties. *Enzyme and microbial technology*, *9*(7), 426-429.

Fontaine, A., Diouf, I., Bakkali, N., Missé, D., Fusai, T., Rogier, C., & Almeras, L. (2011). Implication of haematophagous arthropod salivary proteins in host-vector interactions. *Parasites & vectors*, *4*(1), 1-17.

Ghosh, K., Roy, M., Kar, N., & RingØ, E. (2010). Gastrointestinal bacteria in rohu, Labeo rohita (Actinopterygii: Cypriniformes: Cyprinidae): scanning electron microscopy and bacteriological study. *Acta Ichthyologica et Piscatoria*, *40*(2), 129-135.

Ghoshal, M. (2018). Isolation identification and functional characterization of bacteria from Argulus bengalensis Ramakrishna 1951. Available at: https://shodhgangotri.inflibnet.ac.in/bitstream/20.500.14146/6298/1/256_synopsis.pdf.

Gomes, A. D. C., de Souza, J. M., Bergamaschi, D. P., Dos Santos, J. L., Andrade, V. R., Leite, O. F., ... & de Lima, V. L. (2005). Anthropophilic activity of Aedes aegypti and of Aedes albopictus in area under control and surveillance. *Revista de saude publica*, *39*, 206-210.

Graf, J. (1999). Symbiosis of Aeromonas veronii biovar sobria and Hirudo medicinalis, the medicinal leech: a novel model for digestive tract associations. *Infection and immunity*, *67*(1), 1-7.

Graf, J. (2000). FEATURES-Symbiosis of Aeromonas and Hirudo medicinalis, the Medicinal Leech-The simplicity and accessibility of this digestive-tract association provide an opportunity to investigate its. *ASM News-American Society for Microbiology*, *66*(3), 147-153.

Gresty, K. A., Boxshall, G. A., & Nagasawa, K. (1993). The fine structure and function of the cephalic appendages of the branchiuran parasite, Argulus japonicus Thiele. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *339*(1287), 119-135.

Hesser, E. F. (1960). Methods for routine fish hematology. *The Progressive Fish-Culturist*, *22*(4), 164-171.

Hoffbrand, V., & Steensma, D. P. (2019). *Hoffbrand's essential haematology*. John Wiley & Sons.

Holt, J. G., Krieg, N. R., Sneath, P. H., Staley, J. T., & Williams, S. T. (1994). Bergey's Manual of determinate bacteriology

Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.-Y., and Fukatsu, T. 2010.*Wolbachia*as a bacteriocyte-associated nutritional mutualist. Proceedings of National Academy of Science, United States of America 107, 769–774.

Kar, N., Roy, R. N., Sen, S. K., & Ghosh, K. (2008). Isolation and characterization of extracellular enzyme producing bacilli in the digestive tracts of rohu, Labeo rohita (Hamilton) and Murrel, Channa punctatus (Bloch). *Asian Fisheries Science*, *21*(4), 421-434.

Kaya, H. K., & Gaugler, R. (1993). Entomopathogenic nematodes. *Annual review of entomology*, *38*(1), 181-206.

Kotál, J., Langhansová, H., Lieskovská, J., Andersen, J. F., Francischetti, I. M., Chavakis, T., ... & Chmelař, J. (2015). Modulation of host immunity by tick saliva. *Journal of proteomics*, *128*, 58-68.

Lesel, R., Fromageot, C., & Lesel, M. (1986). Cellulose digestibility in grass carp, Ctenopharyngodon idella and in goldfish, Carassius auratus. *Aquaculture*, *54*(1-2), 11-17.

Lindsay, G. J. H., & Harris, J. E. (1980). Carboxymethylcellulase activity in the digestive tracts of fish. *Journal of fish Biology*, *16*(3), 219-233.

Marshall, W. S., Cozzi, R. R. F., & Strapps, C. (2008). Fish louse Argulus funduli (Crustacea: Branchiura) ectoparasites of the euryhaline teleost host, Fundulus heteroclitus, damage the ion-transport capacity of the opercular epithelium. *Canadian Journal of Zoology*, *86*(11), 1252-1258.

Minard, G., Tran, F. H., Raharimalala, F. N., Hellard, E., Ravelonandro, P., Mavingui, P., & Valiente Moro, C. (2013). Prevalence, genomic and metabolic profiles of Acinetobacter and Asaia associated with field-caught Aedes albopictus from Madagascar. *FEMS microbiology ecology*, *83*(1), 63-73.

Neiffer, D. L., & Stamper, M. A. (2009). Fish sedation, anesthesia, analgesia, and euthanasia: considerations, methods, and types of drugs. *ILAR journal*, *50*(4), 343-360.

Nelson, M. C., & Graf, J. (2012). Bacterial symbioses of the medicinal leech Hirudo verbana. *Gut Microbes*, *3*(4), 322-331.

Pais, R., Lohs, C., Wu, Y., Wang, J., & Aksoy, S. (2008). The obligate mutualist Wigglesworthia glossinidia influences reproduction, digestion, and immunity processes of its host, the tsetse fly. *Applied and environmental microbiology*, *74*(19), 5965-5974.

Pidiyar, V., Kaznowski, A., Narayan, N. B., Patole, M., & Shouche, Y. S. (2002). Aeromonas culicicola sp. nov., from the midgut of Culex quinquefasciatus. *International Journal of Systematic and Evolutionary Microbiology*, *52*(5), 1723-1728.

Pinus, M., & Müller, H. E. (1980). Enterobacteria of bats (Chiroptera)(author's transl). *Zentralblatt fur Bakteriologie. 1. Abt. Originale. A: Medizinische Mikrobiologie, Infektionskrankheiten und Parasitologie*, *247*(3), 315-322.

Poly, W. J. (2008). Global diversity of fishlice (Crustacea: Branchiura: Argulidae) in freshwater. *Hydrobiologia*, *595*(1), 209-212.

Ramakrishna, G. (1951). Notes on the Indian species of the genus Argulus Müller (Crustacea Copepoda) parasitic on fishes. *Records of the Zoological Survey of India*, *49*(2), 207-215.

Ribeiro, J. M. C. (1987). Role of arthropod saliva in blood feeding. *Ann Rev Entomol*, *32*, 463-478.

Saha, S. K., Guha, A., & Banerjee, A. (2011). Feeding apparatus and associated glands in the freshwater fish ectoparasite Argulus Siamensis Wilson, 1926 (Branchiura). *Crustaceana*, *84*(10), 1153.

Schofield, C. J., Dolling, W. R., Lane, R. C., & Crosskey, R. W. (1993). Medical insects and arachnids.

Senthilraj, R., Prasad, G. S., & Janakiraman, K. (2016). Sequence-based identification of microbial contaminants in non-parenteral products. *Brazilian Journal of Pharmaceutical Sciences*, *52*, 329-336.

Stetter, M. D. (2001). Fish and amphibian anesthesia. *Veterinary Clinics of North America: Exotic Animal Practice*, *4*(1), 69-82.

Swanepoel, J.H., Avenant-Oldewage, A., 1992. Comments on the morphology of the preoral spine in *Argulus*(Crustacea: Branchiura). Journal of Morphology 212, 155–162.

Trust, T.J., Sparrow, R.A.H., 1974.The bacterial flora in the alimentary tract of freshwater salmonid fishes.Canadian Journal of Microbiology 20, 1219−1228.

Vaughan, E. E., Heilig, H. G., Ben-Amor, K., & De Vos, W. M. (2005). Diversity, vitality and activities of intestinal lactic acid bacteria and bifidobacteria assessed by molecular approaches. *FEMS Microbiology Reviews*, *29*(3), 477-490.

Walter, H. E. (1984). Proteases and their inhibitors. 2. 15. 2 Method with haemoglobin, casein and azocoll as substrate. *Methods of enzymatic analysis.*, 270-277.